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Extraction and Characterisation of Flavoured Tobacco by GC-MS Studies and Validation of Results by GC-FID to Investigate Hazardous Flavour Ingredients

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Flavoured tobacco is mainly consumed in India and neighbouring countries like Pakistan, Afghanistan, and Nepal and the hazards are known. Considering the need to identify such flavouring ingredients and a simple analytical method was required to quantify such favouring ingredients and hazardous / allergens, we selected top brands available in India for investigation. We simply extracted the ingredients by triturating with Diethyl Ether, evaporating solvent ether and reconstituting the extract in Acetone & Ethanol for GC-MS & GC-FID work respectively. The flavour ingredients were identified, and hazardous ingredients, viz. Diethyl Phthalate was identified. It was found around 2.5% to 3.0%. The GC-MS method was validated with GC-FID analysis with Linearity, LOD & LOQ study.

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1. INTRODUCTION

Flavour refers to the biological, physical, and psychological impacts induced by the interplay of chemical stimulants, fragrances, aromas, and the olfactive systems of living organisms (Augusto) [1]. Flavors that "are prominent in pyrazines including burley tobacco flavours, chocolates, nuts, and reaction/processed flavour" have a "muting influence" on menthol cooling and might generate a "flavour character that is incompatible" (Wayne) [2]. It is widely known that tobacco leaf is the primary raw material for the tobacco industry; its metabolites are strongly associated with the flavour of cigarettes [3]. According to research, those who use flavoured tobacco products are more likely to become "hooked" than someone trying non-flavoured tobacco products for the first time. This is because flavoured tobacco products are frequently seen as "beginning" items. The flavouring disguises the harshness of tobacco products, making them more addictive and more difficult to stop. According to the Centers for Disease Control (CDC), flavoured tobacco is more addictive than traditional tobacco products. Previous study combines chemical analysis and flavour descriptions of flavour additives used in tobacco products, and provides a starting point to build an extensive library of flavour components [4].

In 2009, all flavoured cigarettes except those with menthol were banned by the Food and Drug Administration (FDA). The FDA was also granted the authority to regulate other tobacco products. Several new flavoured, smokeless tobacco products are available, such as energy dip, dry snuff, dip and chew, nicotine dissolvable, e-cigarettes and snus (a tobacco product with few additives like salt & sodium carbonate usually kept below the lips). Despite this, it has enormous economic, agricultural, and social significance. It has been extensively used for smoking, chewing, and sniffing. There are around 600 recognised tobacco species, but only two are consumed by humans [5,6].

Although it originated in South America, tobacco is now grown worldwide and in the Republic of Croatia [7,8]. China is the world's largest producer and consumer of tobacco, with an annual production of 4-5 million. (Hu et al.,2015) [9,10]. Approximately 4000 chemicals, including particles and gases, are present in tobacco, of which 1000 are inhaled while smoking [11]. Nicotine is the most well-known substance found in tobacco leaves and the smoke produced when they are. Our previous research has shown that tobacco includes several beneficial compounds, including nicotine and solanesol, which have strong inhibitors that strongly inhibit *Bacillus subtilis and Micrococcus lysodeikticus* [12] Tobacco is reprocessed due to its ability to absorb moisture to make it appropriate for storage and to meet the spot better meet better or make cigarettes [13]

Several studies have identified distinct classes of chemicals in tobacco, namely alkaloids (including nicotine) [14] aroma compounds [15] fatty alcohols and phytosterols [16]. Due to a large number of distinct chemical compt present in tobacco and tobacco-related materials that may be extracted simultaneously, it is challenging to develop selective extraction techniques for specific molecules. Severson et al. described how the major cuticular components of different commercial tobacco cultivars and tobacco introductions were found [17]. Troje et al. establish a new approach for identifying specific components from two distinct categories of chemicals in the same extract derived from tobacco material in a single step [18].

Eighty-three molecules, including 45 cembranoids, 15 adenoids, 20 sucrose esters, and 3 glucose esters, were identified (or fructose esters). There may be three novel cembranoids and seven new labdanoids. Glucose esters (or fructose esters) are also described for the first time in Nicotiana tobacco [19] Menthol cigarettes are far more addictive than regular cigarettes. Menthol is more popular among youth, women, and minority groups. The California Department of Public Health reports that African American smokers have the greatest prevalence of menthol cigarette use (82.6%). In a few nations, flavour compounds in electronic cigarette (EC) fluids that may harm human health have been examined [20]. Alternatives to traditional cigarettes (CC) have been offered to the public in recent years. The market has seen the introduction of electronic cigarettes (ECIG) and heated tobacco products (HTP) [21].

Water pipe tobacco smoking (WTS, also known as hookah, shisha, narghile, and other names) is

passing tobacco smoke through water before inhaling It [22]. Hookah is a communally smoked water pipe with various delicious flavours. The hookah has the same carcinogens as cigarettes and has been linked to lung cancer, respiratory sickness, low birth weight, and periodontal disease. According to the CDC, an hour of hookah smoking is comparable to inhaling 100–200 times as much smoke as one cigarette. The three types of chewing tobacco are loose leaf, plug, and twist. It has carcinogens and raises the possibility of getting mouth cancer. The CDC says there is a strong link between chewing tobacco, precancerous white patches in the mouth, and irritation-caused gum recession. The County and Statewide Archive of Tobacco Statistics (C-Stats) says that in Sacramento, men (14%) are more likely to chew tobacco than women (7.7%) [23]. As one of the most important non-food crops, tobacco plays a significant role in global agriculture. Tobacco processing enterprises create a vast quantity of tobacco trash as a by-product, often discarded owing to its nicotine concentration; only a tiny portion of this garbage gets recycled [24].

Diethyl phthalate was one of the powerful allelochemicals in barnyard grass root exudates [25] Diethyl phthalate (DEP) is one of the phthalate esters with a short chain and low molecular weight [26]. Diethyl phthalate (DEP), an odourless, colourless, greasy chemical, is utilised to enhance the performance and durability of various products [27]. Because phthalates, including DEP, are not covalently attached to goods, they are easily discharged into the environment and can be taken orally, inhaled, or dermally [28,29]. Two studies assessed foetal survival following exposure to DEP during gestation (NTP 1988) [30,31]. Two further studies assessed the number of newborn pups that survived after gestation [32,33,34].

1.1 Origin of the Research Problem

Cigarette smoking and chewing tobacco have negative health impacts, such as cancer and lung and cardiovascular illnesses. The scenario in India is also becoming worst, Flavoured tobacco brands are becoming popular, and Ghutka is prepared by rubbing catechu, tobacco and flavouring ingredients. This is more hazardous than chewing plain tobacco with calcium hydroxide (Chuna) because manufacturers use a natural essential oil combination for flavouring, and manufacturers use Aroma Chemicals, which are of cheap quality.

1.2 Significance of the Study

The flavour ingredients in Tobacco are of two types –

a) Natural essential oils which are non-hazardous or less hazardous and
b) Synthetic analogues, which are aroma chemicals.

The synthetic aroma chemicals have side effects - allergic disorders affecting the lung, hepatic toxicity, renal toxicity, Neurotoxicity etc. In the proposed study, we will try to identify the flavouring ingredients in tobacco products available on the market. This survey will probe the hazardous chemicals used along with the impurities carried with them. This will help to make the aware FDA and concerned public health officials.

1.3 Objectives

The study's primary purpose is to identify dangerous compounds used in flavoured tobacco and provide a straightforward approach for quantitative analysis. This will help FDA to establish the SOP of the analysis of tobacco & related products.

1.4 Experimental Work Done

The experimental work done is divided into three phases:

1) Extraction of Flavoured Tobacco & similar products
2) GC-MS analysis of Tobacco Extracts, Flavour Concentrates & Identification of hazardous compounds used
3) GC-FID analysis and Limit of Detection, Linearity study by using the reference standard

1) Extraction of Flavoured Tobacco:

Reagent and Materials:

Among several market samples of flavoured tobacco, 3 samples were shortlisted. For the sake of reputation of these companies we have coded these top 3 brands in India as M-1, M-2 & M-3. M-1 was selected as being the most popular & having pleasant & high intensity. The intensity of the flavour was very high and could be smelled from a long distance (20-25 feet).

Solvents & Reference Standards:

Diethyl Ether (MERCK), Absolute Ethanol (MERCK), Sodium Sulfate Anhydrous (MERCK), Diethyl Phthalate (Sigma Aldrich, Lot # LRAC4368), Whatman filter paper No 41

Method of Extraction:

The tobacco leaf samples were triturated/powdered in a granite mortar & pestle. 25 gm of the sample was extracted 6 times with 50 ml of Diethyl Ether, filtered on a multi-folded Whatman filter paper no 41, the fractions were collected together, and the solvent ether was evaporated slowly in a water bath at around 45-50^{o} C. The dark brown-coloured extract was vicious and oily. It was further diluted with Acetone /Absolute Alcohol (Ethanol):

1) For GC-MS analysis with dilution factor 1:10 (Acetone)
2) For GC-FID analysis with dilution as required for Limit of Detection & Linearity study.

The respective diluted solutions were used for:

1) GC-MS analysis for investigation of flavour ingredient molecules
2) GC-FID analysis for quantitative analysis of the ingredient molecules

2) GC-MS Analysis work:

GC-MS analysis of the volatile constituents of tobacco flavour was performed with Agilent GC 7890A series & GC-MS 5977 (Single quadruple). The volatile constituents were separated on a 30 m 0.25 mm i.d., df = 0.25 µm, Rxi-5 Sil (fused silica column). Helium was used as carrier gas at a 1.0 ml/ min flow rate.

The column temperature was held at 50^{o}C for 2 min then programmed as follows:

1) @ 4^{o}C per min to 120^{o}C; this was held for 2 min. Then,
2) Second @ 5^{o}C per min to 260^{o}C, which was also held for 2 min.

The input and ionisation source temperatures were 230°C and 150°C, respectively. In contrast, the temperature of the GC-MS transfer line was 250°C.

Results of GC-MS work:

Interpretation of the NIST library & Internal Library search report is shown in Table 1.

Table 1. GC-MS Analysis Library Search Report Summary

#	Retention Time	Area%	NIST Library identification	Solvent corrected Area
1	1.45	38.23	Acetone	-
2	3.52	0.74	Diacetone Alcohol	1.20
3	6.46	0.76	**Phenol**	1.23
4	7.93	1.40	Benzyl Alcohol	2.27
5	9.39	0.65	Phenyl Ethyl Methyl Ether	1.05
6	10.37	1.29	Phenyl Ethyl Alcohol	2.09
7	10.60	0.79	(4-tert-butylcyclohexyl) acetate	1.28
8	11.79	0.79	Iso Borneol	1.28
9	12.34	11.81	Menthol	19.12
10	12.91	0.64	Beta Pinene	1.04
11	14.15	3.33	Citronellol	5.39
12	15.02	2.95	Geraniol Formate	4.77
13	18.05	0.81	Methyl Anthranilate	1.31
14	18.33	16.59	**Nicotine**	26.86
15	18.60	1.01	Citronellyl Propionate	1.63
16	22.80	1.57	Alpha Guaiene	2.54
17	25.82	0.92	Delta Guaiene	1.49
18	29.36	2.43	**Diethyl Phthalate**	3.93
19	31.74	0.95	Carotol	1.54
20	31.85	3.31	Patchouli Alcohol	5.36
21	37.14	5.65	**Benzyl Benzoate**	9.15
22	40.96	3.38	Musk Tetralin (Tonalid)	5.47

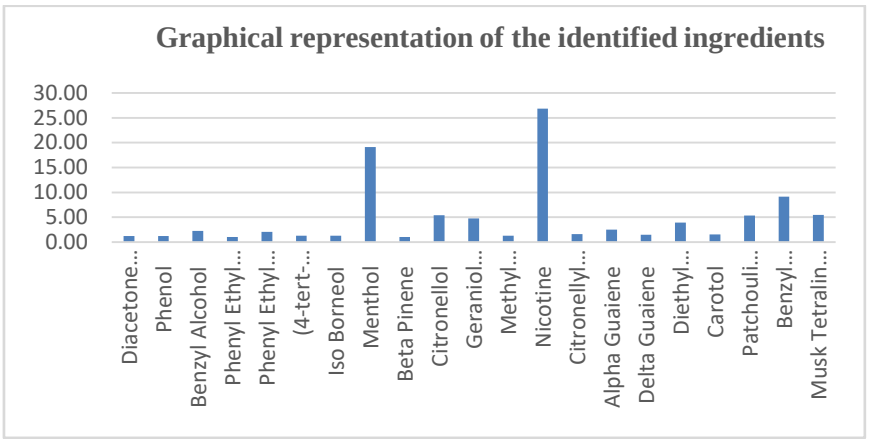

Chart 1. Graphical representation

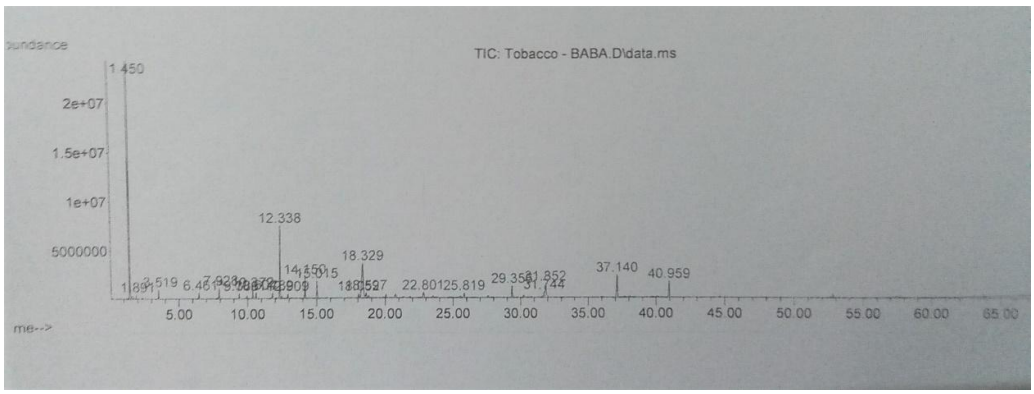

Fig. 1. GC-MS chromatogram

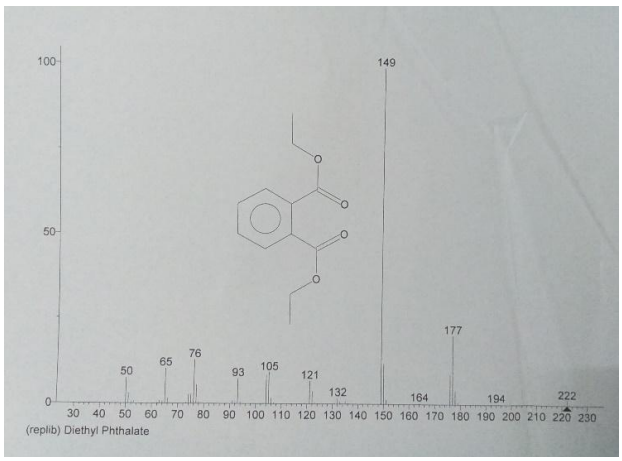

Fig. 2. Mass spectra of diethyl phthalate

After omitting the solvent peak (Acetone), the total compounds (molecules) identified are 21. Among these 21 molecules. The Graphical representation gives an idea of the proportion of the major & minor ingredients in the flavour used as shown below in Chart 1 & Chromatogram in Fig. 1, Mass spectra in Fig. 2.

3) GC-FID analysis, Limit of Detection and Linearity study:

For GC-FID analysis, Limit of Detection (LOD) & Linearity study, the hazardous marker compound identified was 'Diethyl Phthalate'. Reference

standard for Diethyl Phthalate from Sigma-Aldrich (Sigma Aldrich, Lot # LRAC4368) was procured.

A) Preparation of Standard solutions:

Standard solutions from the above reference standard were prepared by using AR grade Absolute Alcohol (Ethanol) as a dilution solvent shown in Table 2:

Table 2. Preparation of standard solutions from a reference standard

#	Standard Solutions
1	DEP Ref Std 0.01% in Ethanol w/v
2	DEP Ref Std 0.02% in Ethanol w/v
3	DEP Ref Std 0.05% in Ethanol w/v
4	DEP Ref Std 0.10% in Ethanol w/v
5	DEP Ref Std 0.25% in Ethanol w/v
6	DEP Ref Std 0.50% in Ethanol w/v

B) Preparation of Sample solutions:

The dark brown-coloured extract of Flavoured Tobacco after the evaporation of Diethyl Ether (solvent) was vicious and oily; it was diluted 10 times (1:10) with AR Grade Absolute Alcohol (Ethanol). This sample solution was injected after the Standard solutions.

Chromatographic Conditions:

GC-FID analysis of the Standard & Sample solutions was performed with Shimadzu GC 2014 series. The column used was the same for GC-MS analysis: 30 m 0.25 mm i.d., df = 0.25 µm, Rxi-5 Sil (fused silica column). Nitrogen was used as carrier gas at a 1.0 ml/ min flow rate.The column temperature was held at 50^{o} C for 2 min then programmed as follows:

1) @ 4^{o} C per min to 120^{o} C; this was held for 2 min. Then,
2) @ 5^{o} C per min to 260^{o} C, which was also held for 2 min.

The Injector temperature was 250^{o} C, and the Detector temperature was 280^{o}C.

The injection volume for six standard solutions & sample solution was 1 µL. six injections for each standard solution were done &% RSD was noted.

2. RESULTS AND DISCUSSION

GC-FID Analysis: Linearity research, Limit of Detection (LOD), and Limit of Quantification (LOQ) Limit of Quantification (LOQ) was the lowest concentration of analytes that the technique could consistently identify with acceptable precision and accuracy. In this analysis, LOQ was based on the lowest concentration in the calibration curve, that is, 0.01% (~ 0.1 mg/ml or 100 ppm). LOD is three times lower than LOQ; this LOD is 0.0033% (~0.033 mg/ml or 33.33 ppm).

2.1 Linearity of Calibration Curve and Working Range

The ability of a technique to achieve an analyte concentration that was proportionate to the measured signal within the operating range was known as linearity. The method's linearity was confirmed by measuring the instrument signal vs the concentration data. The variance of the regression line slope was used to summarise the obtained data. The intercept and slope of a straightforward linear regression equation applied to the data should be used to compute the correlation coefficient. The following is the linear regression equation:

$$Y = mX + C$$

m = Slope of the equation/coefficient
C = y intercept
Y =dependent variable
X =independent variable

Before sample analysis, the instrument performed and analysed six calibration standard points for Diethyl Phthalate concentration. The concentration range of the calibration curve for the six injections was between 0.01% (0.1 mg/ml) to 0.5% (5mg/ml). R^2 of the linear curves is 0.9997 across the calibration range of 0.1 to 5 mg/ml.

2.2 Linear Regression

1. Y and X relationship

R Square (R^2) equals 0.9997. It means that 100% of the variability of Y is explained by X.

Correlation (R) equals 0.9999. It means a very strong direct relationship between X and Y is shown in Fig. 3.

Thus, the sample concentration was calculated from the above calibration curve. The Area under peak was 255529, and the concentration calculated using the curve was 0.21% (2.1 mg/ml). Considering the dilution factor, DEP content is 2.1% of DEP (Diethyl Phthalate).

Table 3. The concentration range of the calibration curve of DEP

% Concentration	Average Area under peak	Average% Area	% RSD
0.01	11829	0.0103	0.0083
0.02	26088	0.0218	0.0029
0.05	65674	0.0534	0.0011
0.10	124753	0.1001	0.0006
0.25	302196	0.2593	0.0003
0.50	617363	0.5075	0.0001

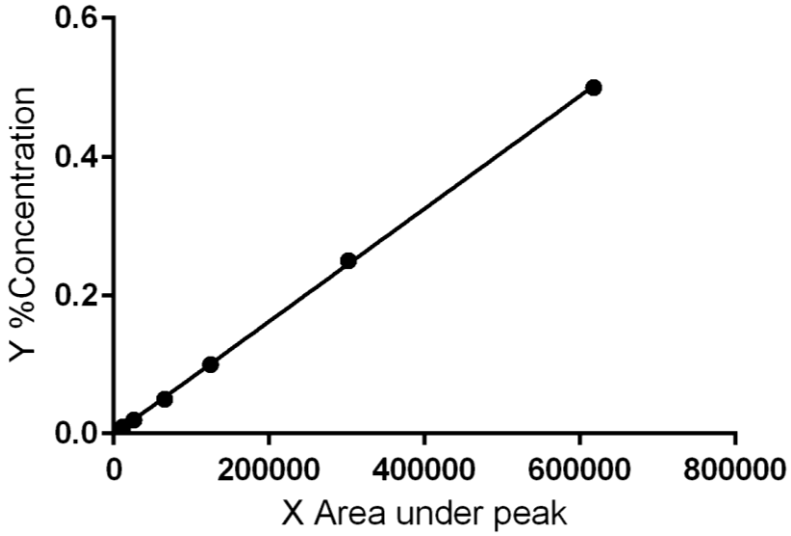

Fig. 3. Linearity curve, Relationship between concentration and area under the peak

The method is validated concerning resolution, reliability & reproducibility. Y and X relationship R Square (R^2) equals 0.9998. It means that 100% of the variability of Y is explained by X. correlation (R) equals 0.9999. It means that there is a very strong direct relationship between X and Y. This method can be used for the estimation of Diethyl Phthalate in Flavoured Tobacco.

3. CONCLUSION

Looking at the analysis of GC-MS & GC-FID, we can conclude that the GC method for estimating Diethyl Phthalate is validated.

Phenol which is also detected by GC-MS data is considered to be quite toxic to humans via oral exposure. Anorexia, progressive weight loss, diarrhea, vertigo, salivation, a dark coloration of the urine, and blood and liver effects have been reported in chronically (long-term) exposed humans [34]. The toxicity data of the ingredient, Benzyl Benzoate, needs to be verified because it can cause ataxia, convulsions & respiratory paralysis. This extraction and GC analysis method can be used to estimate Diethyl Phthalate.

Diethyl Phthalate is undesirable and causes many health hazards. It aggravates pulmonary function and inflammation of the airway in asthma patients, develops infertility, and its carcinogenic activity is suspicious.

Diethyl Phthalate is mainly used for the dilution of fragrance oil. In food flavours, Triethyl Citrate is preferred due to its non-hazardous nature. The trend of using Diethyl Phthalate could be because of the price difference and high solubility of the ingredients in DEP.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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